# BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

## IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.**

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/00 — A2

(11) International Publication Number: **WO 98/52590**

(43) International Publication Date: 26 November 1998 (26.11.98)

(21) International Application Number: PCT/CA98/00448

(22) International Filing Date: 11 May 1998 (11.05.98)

(30) Priority Data:
2,205,771 22 May 1997 (22.05.97) CA

(71) Applicant *(for all designated States except US)*: HYAL PHARMACEUTICAL CORPORATION [CA/CA]; 2425 Skymark Avenue, Mississauga, Ontario L4W 4Y6 (CA).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: TURLEY, Eva, Anne [CA/CA]; 104–234 St. Clair Avenue West, Toronto, Ontario M4V 1R4 (CA).

(74) Agent: HUGHES, Etigson; Suite 200, 175 Commerce Valley Drive West, Thornhill, Ontario L3T 7P6 (CA).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: IMPROVED DELIVERY OF DISEASE MODIFIERS

(57) Abstract

A pharmaceutical composition component comprising hyaluronan binding motif (HBM) interposed between a form of hyaluronan having a molecular weight (protein standard) less than 750'000 daltons and a disease modifier which comprises a peptide or protein.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **AL** | Albania | **ES** | Spain | **LS** | Lesotho | **SI** | Slovenia |
| **AM** | Armenia | **FI** | Finland | **LT** | Lithuania | **SK** | Slovakia |
| **AT** | Austria | **FR** | France | **LU** | Luxembourg | **SN** | Senegal |
| **AU** | Australia | **GA** | Gabon | **LV** | Latvia | **SZ** | Swaziland |
| **AZ** | Azerbaijan | **GB** | United Kingdom | **MC** | Monaco | **TD** | Chad |
| **BA** | Bosnia and Herzegovina | **GE** | Georgia | **MD** | Republic of Moldova | **TG** | Togo |
| **BB** | Barbados | **GH** | Ghana | **MG** | Madagascar | **TJ** | Tajikistan |
| **BE** | Belgium | **GN** | Guinea | **MK** | The former Yugoslav Republic of Macedonia | **TM** | Turkmenistan |
| **BF** | Burkina Faso | **GR** | Greece | | | **TR** | Turkey |
| **BG** | Bulgaria | **HU** | Hungary | **ML** | Mali | **TT** | Trinidad and Tobago |
| **BJ** | Benin | **IE** | Ireland | **MN** | Mongolia | **UA** | Ukraine |
| **BR** | Brazil | **IL** | Israel | **MR** | Mauritania | **UG** | Uganda |
| **BY** | Belarus | **IS** | Iceland | **MW** | Malawi | **US** | United States of America |
| **CA** | Canada | **IT** | Italy | **MX** | Mexico | **UZ** | Uzbekistan |
| **CF** | Central African Republic | **JP** | Japan | **NE** | Niger | **VN** | Viet Nam |
| **CG** | Congo | **KE** | Kenya | **NL** | Netherlands | **YU** | Yugoslavia |
| **CH** | Switzerland | **KG** | Kyrgyzstan | **NO** | Norway | **ZW** | Zimbabwe |
| **CI** | Côte d'Ivoire | **KP** | Democratic People's Republic of Korea | **NZ** | New Zealand | | |
| **CM** | Cameroon | | | **PL** | Poland | | |
| **CN** | China | **KR** | Republic of Korea | **PT** | Portugal | | |
| **CU** | Cuba | **KZ** | Kazakstan | **RO** | Romania | | |
| **CZ** | Czech Republic | **LC** | Saint Lucia | **RU** | Russian Federation | | |
| **DE** | Germany | **LI** | Liechtenstein | **SD** | Sudan | | |
| **DK** | Denmark | **LK** | Sri Lanka | **SE** | Sweden | | |
| **EE** | Estonia | **LR** | Liberia | **SG** | Singapore | | |

## TITLE OF INVENTION

Improved Delivery of Disease Modifiers

## FIELD OF INVENTION

This invention relates to novel methods for delivery of disease modifiers using hyaluronan and to new compositions comprising the disease modifiers and hyaluronan.

## BACKGROUND OF INVENTION

Hyaluronic acid is a large, complex oligosaccaride consisting of up to 50,000 pairs of the basic disaccharide glucuronic acid-ß(1-3) N-acetylglucos-amine ß(1-4). It is found *in vivo* as a major component of the extracellular matrix. Its tertiary structure is a random coil of about 50 nm in diameter. Hyaluronic acid appears in nature in its sodium salt form. Hyaluronic acid and its pharmaceutically tolerable or acceptable salts (such as sodium hyaluronate) are referred to as Hyaluronan (HA).

Hyaluronan has the ability to bind a large amount of water, which *in vivo* makes it a viscous hydrated gel with viscoelastic properties. It is found in this form in the mammalian eye, both in the vitreous and in the extracellular matrix.

Hyaluronan (Hyaluronic Acid and pharmaceutically acceptable Salts Thereof) have been disclosed for use with medicine and/or therapeutic agents for the treatment of disease and/or conditions (see PCT Application, PCT/CA 90/00306, International Publication No. WO 91/04058). Subsequent applications taught the combination of hyaluronic acid and pharmaceutically acceptable salts thereof for topical treatment and for accumulation (see PCT Application, PCT/CA 93/00061, International Publication No. WO 93/16732). It has been postulated that the medicine or therapeutic agent for example, an NSAID, appears to be associated with the hyaluronan as a clathrin (term is taken from clathrinida, an order of sponges which have an asconoid structure and lack a true dermal membrane or cortex), or is associated with the hyaluronan in a patient to whom the combination is administered in association with the patient's serum albumin which serum albumin appears to bind to the hyaluronan.

It is possible to bind hyaluronan directly to a medicine or therapeutic agent. In this regard, see *"Effects of Precipitates formed by insulin with hyaluronic acid and mucoid from vitreous humor in depressing blood-sugar levels"*, Science 1950; 111: 520-521 at 520; *"Reaction of Cationic Groups of Chlorpromazine with Anionic Macromolecules:*

*Complexes with DNA, RNA, Hyaluronic Acid and Heparin"*, Acta pharmacol. et toxicol 1974, 34, 27-32 at pages 30 to 31, and U.S. Patent 5,166,331.

U.S. Applications Serial No. 08/486,328 and 08/520,591 and PCT Application PCT/CA95/00477, also owned by Hyal Pharmaceutical Corporation, teach the modulation of cellular activity of tissue and cells expressing a high affinity cell-surface receptor for hyaluronic acid by the use of forms of hyaluronic acid. These cell surface receptors comprise adhesion molecule CD44 and adhesion molecule HARLEC (Hyaluronic Acid [Hyaluronan] Receptors Liver Endothelial Cells) and regulatory molecule RHAMM (Receptor for HA Mediated Motility), for binding hyaluronan. HARLEC is expressed (produced and put on the cell surface) in liver endothelial cells. The administration of an effective amount of a form of hyaluronic acid to bind with the cell-surface receptors modulates cellular activity of tissues and/or cells expressing such high affinity cell-surface receptors for hyaluronic acid (for example, an adhesion or regulatory molecule) in the human body.

One of the reasons why the hyaluronic acid is able to be used to transport the medicine and/or therapeutic agent is its selective binding to the cell-surface receptors through a Hyaluronan Binding Motif. Hyaluronan Binding motif (HBM) has been identified and is identified BX7B. See the article entitled *Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein;* The EMBO Journal, Vol. 13, No. 2 (1994) pp. 286-296.

While disease modifiers such as Cytokines, peptides mimicking cytokines, and proteins mimicking cytokines for example, may be administered to humans with the subject matter of PCT/CA 90/00306 (International Publication Number WO 91/04058), we have developed an improved method of administration of these disease modifiers (cytokines, peptides mimicking cytokines and proteins mimicking cytokines and other proteins and peptides).

Hyaluronan interacts at sites of cell migration and proliferation via specific hyaluronan receptors (CD44, RHAMM, CD38, TSG-6, and extra-cellular hyaluronan binding proteins (Versican, Aggrecan, Perlican, link-protein, GHAP) all being examples thereof). These receptors and binding proteins are upregulated at the cites of proliferation/migration. As a

result, addition of exogenous hyaluronan targets to these sites for example, where injury has occurred.

It is therefore an object of this invention to provide a new method of delivery of disease modifiers to the human using hyaluronan.

It is a further and other object of the invention to provide new compositions for use in the new method of delivery.

These and other objects of the invention will be realized by those skilled in the art from the following summary of the invention and detailed description of embodiments thereof.

## SUMMARY OF INVENTION

Hyaluronan binding motif (HBM) as previously stated has (have) been identified in all hyaluronan binding proteins and receptors and is strongly conserved amongst species. HBM is identified as BX7B and is known to persons skilled in the art. The affinity of the hyaluronan for the HBM is enhanced if flanking basic amino acids are added or if several internal basic amino acids are added at position 4,5. Furthermore, binding activity is enhanced if the intervening amino acids are hydrophobic and not acidic. Using the hyaluronan binding motif (HBM), it is now possible to bestow hyaluronan binding properties upon a protein or peptide that does not normally bind to hyaluronan. For example, the addition of the HBM to casein, a milk protein that does not bind to hyaluronan bestows binding activity on the protein.

Therefore, according to one aspect of the invention, I propose the use of the hyaluronan binding motif (HBM) to be interposed between a disease modifier (which may be a protein or peptide and which protein or peptide one skilled in the art would not consider under normal conditions to be capable of being bound effectively with hyaluronan) and hyaluronan. By linking the components, I have found that the combination will target the disease modifier for example, proteins such as cytokines, peptides mimicking cytokines, and proteins mimicking cytokines to the sites of, for example, injury or disease. The hyaluronan by binding through HBM to the protein or peptide also protects the protein or peptide from attack from Proteases which appear in high numbers at the sites of injury. Further, the hyaluronan also protects protein/peptide from immune system recognition and attack and possible destruction.

While the disease modifier includes proteins and peptides (which may be considered drugs in the usual sense such as for example antibiotics), they also include any other disease modifier which could be chemically linked to the amino or carboxy terminus of the hyaluronan binding peptide. Such disease modifiers include smyonpford. cyclosporin and other therapeutic peptides such as cytokine peptides, or cell adhesion peptides but are not limited thereto. They also include the following:

| Drug | Use | Route |
|---|---|---|
| **Protein Hormones** | | |
| Follicle Stimulating Hormone (FSH) | | |
| Leutinizing Hormone (LH) | amenorrhea | intravenous |
| Prolactin | chronic renal insufficiency | |
| Human Growth Hormone (GH) | human growth hormone deficiency in children | intramuscular parenteral |
| Adrenocorticotropin Hormone (ACTH) and analogues (eg. Leuprolide Acetate) | hypercalcemia inflamation diagnosis of adrenal insufficiency | intravenous |
| Vasopressin, Lypressin Desmopressin | diabetes insipidus | intranasal parenteral injection |
| Oxytocin (OT) | lactation postpartum bleeding induction of labour | intranasal, intravenous parenteral |
| Gonadotropin Releasing Hormone ($G_nRH$) | infertility, suppression of ovulation, prostate and breast tumours | intranasal injection |

| | | |
|---|---|---|
| Gonadorelin | diagnosis of hypothalemic - pituitary - gonadal dysfunction amenorrhoea infertility | subcutaneous, intravenous, intranasal |
| Corticotropin Releasing Hormone (CRH) | | |
| Thyrotropin Releasing Hormone (TRH) | lactation | transdermal, oral |
| Leutinizing Hormone Releasing Hormone (LHRH) | cryptorchidism endometriosis | intranasal |
| Melanocyte - Stimulating Hormone Inhibiting Factor (MIF) | depression Tardive dyskinesia | oral |
| Melanocyte - Stimulating Hormone Releasing Factor (MSH) | | transdermal |
| Growth Hormone Releasing Hormone (GHRH) | | transdermal |
| Somatostatin | acromegaly, GI tumours, gastric ulcers | intranasal intravenous |
| Corticotrophin | diagnostic agent to investigate adrenocortical insufficiency | intravenous |
| Tetracosactide | diagnostic agent to investigate adrenocortical insufficiency | intramuscular intravenous |
| Octreotide Acetate | gastrointestinal endocrine tumours acromegaly | subcutaneous |

| | | |
|---|---|---|
| Parathyroid Hormone (PTH) | osteoporosis | subcutaneous |
| Thyroid Stimulating Hormone (TSH)/ Thyroid Releasing Hormone (TRH) | diagnosis of thyroid disease | injection |
| Insulin | diabetes mellitus | intravenous<br>transdermal |
| Glucagon | hypocalcemia | parenteral<br>intravenous<br>intramuscular |
| Cholecystokinin | chronic pancreatitis, appetite, postoperative paralytic ileus | intranasal, transdermal intravenous |
| Gastrin | | |
| Secretin | | |
| $\alpha_1$ - Antitrypsin | congenital $\alpha_1$ - antitrypsin deficiency | parenteral |
| Trypsin | GI disorders<br>debridement of wounds<br>oedema and inflammation associated with infection or trauma | topical<br>oral |
| Pepsin | digestive aid in pepsin deficiency<br>gastric hypochlorhydria<br>dyspepsia<br>GI disorders | oral |
| Neurotensin (NT) | gastric juice secretion | intravenous |
| Calcitonin (CT) | Paget's disease of bone<br>osteoporosis<br>hypercalcemia<br>gastric secretion<br>intractable pain | intranasal,<br>subcutaneous,<br>intramuscular<br>intravenous<br>oral, parenteral, transdermal |

| | | |
|---|---|---|
| Human Chorionic Gonadotropin (hcG) | cryptorchidism<br>induction of ovulation | intravenous, parenteral |
| Relaxin | facilitates birth<br>scleroderma | |
| **Therapeutic Cytokines** | | |
| Interferons | | |
| IFNα-2a | Kaposi's sarcoma | parenteral |
| IFNα-2b | genital warts<br>Kaposi's sarcoma<br>AIDS-related hairy cell leukemia | parenteral |
| IFNα-n3 | | parenteral |
| IFNβ-1b | Multiple Sclerosis (relapsing, remitting type) | |
| IFNγ-1b | chronic granulomatous disease | parenteral |
| Tumour Necrosis Factor TNFα, TNFβ | | |
| Interleukins | | |
| IL-1β | protection against the effects of radiation and chemotherapeutic agents | parenteral |
| IL-2 | renal cell carcinoma | |
| IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 | | |

| | | |
|---|---|---|
| **Hemotopoietic Proteins** | | |
| Erythropoietin (EPO) | dialysis anemia<br>chemotherapy-induced anemia<br>chemotherapy-induced neutropenia | parenteral |
| Granuloycyte - Colony Stimulating Factor (G-CSF) | bone marrow transplant | parenteral |
| Granulocyte Macrophage - Colony Stim. Fac. (GM-CSF)<br>Macrophage-Colony Stimulating Factor (M-CSF) | bone marrow transplant | parenteral |
| **Growth Factors** | | |
| Epidermal Growth Factor (EGF)<br>Transforming Growth Factor α(TGFα) | corneal and cateract surgeries | topical |
| Platelet-derived growth factor (PDGF) | diabetic and decubitus ulcers<br>wound healing | |
| Transforming Growth Factorβ (TGFβ) | | |
| Fibroblast Growth Factor - basic FGF - acidic FGF | neuropathic ulcers<br>pressure sores | |
| Insulin-like Growth Factor 1 ($IGF_1$) | nutritional support/metabolisn and type II diabetes<br>osteoporosis | |
| Insulin-like Growth Factor2 ($IGF_2$) | | |

| | | |
|---|---|---|
| Nerve Growth Factor | peripheral neuropathies | |
| Skeletal Growth Factor | | |
| **Cardiovascular Therapeutic Proteins** | | |
| **Proteins of the Blood Coagulation Pathway** | | |
| Factor VIII | Hemophilia A | parenteral |
| Factor IX | | |
| Factor VII/VIIa | | |
| Factor XII | | |
| Tissue Factor (Factor III) | | |
| Protein C | | |
| Antithrombin III | | |
| **Fibrinolytic Therapeutic Proteins** | | |
| Tissue Plasminogen Activator | acute myocardial infarction | parenteral |
| Streptokinase and Anisoylated Streptokinase | thromboembolism | injection |
| Fibrolase | myocardial infarction | |
| Urokinase and Single-Chain Urokinase | | parenteral |
| Kidney Plasminogen Activator | | |
| **Angiotensin-Converting Enzyme Inhibitors** | | |
| Captopril | hypertension | oral |
| Enalapril | heart failure | |

| | | |
|---|---|---|
| **Vaccines** | Active Immunization | injection or oral |
| Hepatitis B virus surface antigens | | |
| Influenza virus surface antigens | | |
| Plasmodium surface antigens | | |
| Mycobacterium surface antigens | | |
| Schistosoma surface antigens | | |
| Herpes simplex virus surface antigens | | |
| Trypanosoma surface antigens | | |
| Streptococcus surface antigens | | |
| Epstein - Barr virus surface antigens | | |
| HTLV III virus surface antigens | | |

| | | |
|---|---|---|
| **Therapeutic and Diagnostic Antibodies** | | |
| Murine Native & Radioimmuno-conjugate Antibodies & Fragments | | |
| - Muromonab-CD3 | heart, kidney & liver transplant rejection | parenteral |
| - Staumonab pendetide | detection, staging, and follow-up of colorectal and ovarian cancers | |
| Murine Chemoimmuno-conjugate Antibodies | | |
| Murine Immunotoxin Antibodies & Fragments | | |
| Nonmurine Polyclonal Antibodies | | |
| Human Antibodies & Fragments | | |
| Murine/Human Monoclonal Antibodies & Fragments | | |
| **Opioids** | | |
| β-Endorphin | cancer pain<br>childbirth pain<br>narcotic abstinence syndrome | intravenous<br>intrathecal |
| Dermorphin | | |
| Dynorphins | | |
| Enkephalins | | |
| **Other Proteins** | | |

| | | |
|---|---|---|
| Cylosporine | immunosuppression in allogenic transplants | intravenous |
| Delta Sleep-inducing peptide (DSIP) | Insomnia | intravenous |
| Bestatin | cancer therapy | oral |
| Bacitracin | bacterial infection | topical |
| Gramicidin | bacterial infection | topical |
| Terprotide | hypertension | parenteral |
| Serum Thymide Factor (FTS) | immune deficiencies | intravenous |
| Crude Thymosin | autoimmune disorders | parenteral |
| | collagen vascular disease | intramuscular |
| | chemotherapy | intravenous |
| | rheumatoid arthritis | intravenous |
| Angiogenin | induces formation of blood vessels | |
| Albumin & Plasma Proteins | blood volume replacement | |
| Atrial Natriuretic Factor | fluid and electrolyte homoeostasis<br>regulation of blood pressure | |
| Renin | arterial pressure control | |
| Superoxide Dismutase | inflammation<br>rheumatoid arthritis<br>protection against radiation therapy | |
| Glucocerebrosidase (Algucerase) | Gaucher's Disease | |
| rh DNase | Cystic Fibrosis | |
| Aprotinin | haemorrhage associated with raised plasma concentrations of plasmin | intravenous |

| | | |
|---|---|---|
| Protamine | neutralize effect of heparin | intravenous |
| Asparaginase | induction of remission in acute lymphoblastic leukemia | parenteral |
| rhBMP-2 | bone and cartilage repair | |
| F(ab) fragment | Digoxin overdose | injection |

Other disease modifiers may also be used with this invention. These may include:

Anti microbial Peptides

**Gramicidin and Related Peptides**
(eg. Gramicidin S, Tyrocidines, Gratisin)

**β-lactam and β-lactam like Antibiotics**
Sulfazecin Type (eg. Sulfazecin, Isosulfazecin)
Carbapenem Type
Cephabacin Type (eg. Chitinovorin, Cephabacin)
Norcardicin Type (eg. Formacidin)
Lactivicin Type (eg. Lactivicin)

**Glycopeptide Antibiotics of the Vancomycin Group**
Vancomycin Type (eg. Vancomycin, Orienticin, Eremomycin)
Actinoidin Type (eg. Actinoidin, Avoparcin, Chloropolysporin)
Ristocetin Type (eg. Ristocetin, Actaplanin)
Teicoplanin Type (eg. Teicoplanin, Ardacin, Kibdelin, Parvodicin)

**N-methylated Peptides and Peptolides**
Linear Peptides (eg. Stenothricin)
Cyclic Peptides (eg. Ilamycins, cyclosporins)
Diketopiperazines (eg. Gliotoxin)
True Depsipeptides (eg. Enniatins, Beauvericin)
Actinomycins

**Sideromycins** (eg. Grisein, Albomycin, Desferrioxamine B)
- use iron chelation therapy for removal of excessive iron resulting from genetic defects (primary hemochromatosis, anemias) or repeated blood transfusions

**Phleomycin - Like Antibiotics (eg. Bleomycin)**
- use - squamous cell carcinomas, Hodgkin's lymphomas, testis tumour

**Protease Inhibitors**

**Inhibitors Against Endopeptidases**

**Serine and Cysteine Proteinase Inhibitors**
Leupeptin - use - fertilization, inflammation, chemical carcinogenesis, burns, pancreatitis, muscular dystrophy, antoimmune diseases
Antipain - use - fertilization, inflammation, chemical carcinogenesis, muscular dystrophy
Chymostatin - use - fertilization, inflammation
Elastatinal - use - inflammation
Ac-Leu-Argal
Lystatin
Poststatin

**Aspartic Proteinase Inhibitors**
Pepstatin - use - inflammation, hypertension, ascites and pleural fluid
Pepstanone
Hydroxypepstatin

**Metal Proteinase Inhibitors**
Phosphoramidon - use - inflammation
Steffimycins B & D

**Inhibitors Against Exopeptidases**
**Aminopeptidases Inhibitors**
Amastatin
Actinonin - use - immunopotentiation, analgesia

Arphamenines A & B - use - immunopotentiation, analgesia, autoimmune diseases

Bestatin - use - immunopotentiation, analgesia, hypertension, malignant diseases, muscular dystrophy

Ebelactones A & B - use - immunopotentiation

Formestin

Probestin

Prostatin

Leuhistin

**Dipeptidylamino Peptidase Inhibitors**

Ac-Leu-Argal

Antipain

Leupeptin

Diprotins A & B - use - immunopotentiation

Octastatins A & B

**Carboxy Peptidase Inhibitors**

(S)-α-Benzylmalic acid - use - immunopotentiation

Histargin - use - immunopotentiation, hypertension

**Dipeptidylcarboxy Peptidase Inhibitors**

EDDS

Foroxymithine - use - immunopotentiation, hypertension

Histargin

**Inhibitors Against Plasma-Membrane-Located-Enzymes**

Forphenicine - use - immunopotentiation, muscular dystrophy, malignant diseases

Forphenicinol - use - immunopotentiation, muscular dystrophy, malignant diseases

Esterastin - use - immunopotentiation, inflammation, auto immune diseases

Ebelactone A & B

Thus, I have provided a new composition comprising hyaluronan, hyaluronan binding motif (HBM), for example, found in a hyaluronan binding protein or receptor, and disease modifier (including a drug and/or

therapeutic agent) which can be chemically linked or bound by the HBM (eg. at the amino or carboxy terminus of the hyaluronan binding peptide) to the disease modifier. Thus, the hyaluronan indirectly binds through the hyaluronan binding motif (HBM) to the disease modifier such as a drug which is a protein such that the hyaluronan binding motif (HBM) is interposed between the hyaluronan and the disease modifier.

The new composition (or compound) may be used and administered in manners previously described for example, intravenously, interarterially, interperitoneally, intrapleurally, into the skin, applied topically onto the skin for penetration into the skin, to the oral mucosa, rectally or by direct injection into a tumour, abscess, or similar disease focus or put on a patch to be secured to the skin of the patient or administered via an enema.

Many forms of hyaluronan may be suitable although those preferred are those discussed hereinafter:

One form of hyaluronic acid and/or pharmaceutically acceptable salts thereof (for example sodium salt) suitable for use with my invention is an amount having the following specifications/characteristics:

| TESTS | SPECIFICATIONS | RESULTS |
|---|---|---|
| pH | 5.0 to 7.0 at 25 degrees C. | 6.0 |
| Specific Gravity | 0.990 to 1.010 at 25 degrees C. | 1.004 |
| Intrinsic Viscosity | 4.5 to 11.0 dL/g. | 7.07 |
| Molecular Weight | 178,000 to 562,000 daltons (protein standard) | 319,378 daltons |
| Sodium Hyaluronate | 9.0 to 11.0 mg/mL. Positive | 9.9 mg/ML |
| Assay and Identification | | Positive |

Another such amount may comprise:

| | TESTS | SPECIFICATIONS |
|---|---|---|
| 1. | Description | White or cream odourless powder |
| 2. | Identification (IR Spectrum) | Conforms to Ref. Std. Spectrum |
| 3. | pH (1% solution) | 5.0 to 7.0 |
| 4. | Loss on Drying | NMT 10% |
| 5. | Residue on Ignition | 15.0% to 19.0% |
| 6. | Protein Content | NMT 0.1% |
| 7. | Heavy Metals | NMT 20 ppm |
| 8. | Arsenic | NMT 2 ppm |
| 9. | Residual Solvents | |

| | | |
|---|---|---|
| | a) Formaldehyde | NMT 100 ppm |
| | b) Acetone | NMT 0.1% |
| | c) Ethanol | NMT 2.0% |
| 10. | Sodium Hyaluronate Assay (dried basis) | 97.0 to 102.0% |
| 11. | Intrinsic Viscosity | 10.0 to 14.5 dL/g |
| 12. | Molecular Weight | 500,000 to 800,000 daltons |
| 13. | Total Aerobic Microbial Count (USP 23) | NMT 50 microorganisms/g |
| 14. | Escherichia coli (USP 23) | Absent |
| 15. | Yeasts and Moulds (USP 23) | NMT 50 microorganisms/g |
| 16. | Bacterial Endotoxins (LAL) (USP 23) | NMT 0.07 EU/mg |

Another such amount is available from Hyal Pharmaceuticals Limited and comes in a 15 ml vial of Sodium hyaluronate 20mg/ml (300mg/vial - Lot 2F3). The sodium hyaluronate amount is a 2% solution with a mean average molecular weight of about 225,000. The amount also contains water q.s. which is triple distilled and sterile in accordance with the U.S.P. for injection formulations. The vials of hyaluronic acid and/or salts thereof may be carried in a Type 1 borosilicate glass vial closed by a butyl stopper which does not react with the contents of the vial.

The amount of hyaluronic acid and/or salts thereof (for example sodium salt) may also comprise the following characteristics:

a purified, substantially pyrogen-free amount of hyaluronic acid obtained from a natural source having at least one characteristic selected from the group (and preferably all characteristics) consisting of the following:

i) a molecular weight within the range of 150,000-225,000;
ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;
iii) less than about 0.6% protein on a total weight basis;
iv) less than about 150 ppm iron on a total weight basis;
v) less than about 15 ppm lead on a total weight basis;
vi) less than 0.0025% glucosamine;
vii) less than 0.025% glucuronic acid;
viii) less than 0.025% N-acetylglucosamine;
ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9. Preferably, the hyaluronic acid is mixed with sterile water and the amount of hyaluronic acid has a mean average molecular weight within the range of 150,000-225,000 daltons (protein standard). More preferably, the amount of hyaluronic acid comprises at least one characteristic selected from the group (and preferably all characteristics) consisting of the following characteristics:

i) less than about 1% sulphated mucopolysaccharides on a total weight basis;

ii) less than about 0.4% protein on a total weight basis;

iii) less than about 100 ppm iron on a total weight basis;

iv) less than about 10 ppm lead on a total weight basis;

v) less than 0.00166% glucosamine;

vi) less than 0.0166% glucuronic acid;

vii) less than 0.0166% N-acetylglucosamine;

viii) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7

Applicants may also use sodium hyaluronate produced and supplied by LifeCore™ Biomedical, Inc., having the following specifications:

| Characteristics | Specification |
|---|---|
| Appearance | White to cream colored particles |
| Odor | No perceptible odor |
| Viscosity Average Molecular Weight | < 750,000 Daltons |
| UV/Vis Scan, 190-820nm | Matches reference scan |
| OD, 260nm | < 0.25 OD units |
| Hyaluronidase Sensitivity | Positive response |

| | |
|---|---|
| IR Scan | Matches reference |
| pH, 10mg/g solution | 6.2 - 7.8 |
| Water | 8% maximum |
| Protein | < 0.3 mcg/mg NaHy |
| Acetate | < 10.0 mcg/mg NaHy |

Heavy Metals, maximum ppm

| As | Cd | Cr | Co | Cu | Fe | Pb | Hg | Ni |
|---|---|---|---|---|---|---|---|---|
| 2.0 | 5.0 | 5.0 | 10.0 | 10.0 | 25.0 | 10.0 | 10.0 | 5.0 |

| | |
|---|---|
| Microbial Bioburden | None observed |
| Endotoxin | < 0.07EU/mg NaHy |
| Biological Safety Testing | Passes Rabbit Ocular Toxicity Test |

Another amount of sodium hyaluronate proposed to be used is sold under the name Hyaluronan HA-M5070 by Skymart Enterprises, Inc. having the following specifications:

Specifications' Test Results

| | |
|---|---|
| Lot No. | HG1004 |
| pH | 6.12 |
| Condroitin Sulfate | not detected |
| Protein | 0.05% |
| Heavy Metals | Not more than 20 ppm |
| Arsenic | Not more than 2 ppm |
| Loss on Drying | 2.07% |
| Residue on Ignition | 16.69% |
| Intrinsic Viscosity | 12.75 dl/s (XW: 679,000) |
| Nitrogen | 3.14% |
| Assay | 104.1% |
| Microbiological Counts | 80/g |
| E. coli | Negative |
| Mold and Yeast | Not more than 50/g |

Other forms of hyaluronic acid and/or its salts may be chosen from other suppliers and those described in prior art documents provided they are suitable.

The following references teach hyaluronic acid, sources thereof, and processes for the manufacture and recovery thereof which may be suitable.

As there is no toxicity of the form of hyaluronic acid, the form of hyaluronic acid may be administered in doses in excess of 12mg/kg of body weight, for example, in excess of 1000mg/70kg person and even up to amounts of 3000mg/70kg person without adverse toxic effects. Lower amounts may include 10-50mg of hyaluronan. Exemplary amounts of Hyaluronan used may be 3-10mg of hyaluronan (HA)/kg of body weight of the patient wherein the molecular weight (protein standard) is less than 750,000 daltons.

Many forms of hyaluronan may be suitable for use herein although those preferred are those discussed hereinafter. Particularly, molecular weights of forms of hyaluronan between about 150,000 daltons and about 750,000 daltons (protein standard) in sterile water prepared having a viscosity for intravenous administration are suitable.

One specific form of pharmaceutical grade is a 1% sterile sodium hyaluronate solution (50 ml vials) provided by Hyal Pharmaceutical Corporation which has the following characteristics:

| Tests | Specifications |
|---|---|
| 1. Container Description | 1 50 mL Flint glass vial with a red or gray rubber stopper and an aluminum seal, 20 mm in size. |
| 2. Product Description | A clear, colourless, odourless, transparent, slightly viscous liquid. |
| 3. Fill Volume | 50.0 to 52.0 mL |
| 4. pH | 5.0 to 7.0 at 25 degrees C. |
| 5. Specific Gravity | 0.990 to 1.010 at 25 degrees C. |
| 6. Intrinsic Viscosity | 4.5 to 11.0 dL/g |
| 7. Molecular Weight | 178,000 to 562,000 daltons |
| 8. Sodium Hyaluronate Assay and Identification | 9.0 to 11.0 mg/mL. Positive |
| 9. Particulate Matter | No visible Particulate Matter |
| 10. Sterility | Meets Requirements for Sterility, USP 23 |
| 11. Bacterial Endotoxins (LAL) | Meets Requirements for Bacterial Endotoxins, USP 23. |

This pharmaceutical grade 1% sterile solution of hyaluronan may be made from granules/powder having the following characteristics:

| Tests | Specifications |
|---|---|
| 1. Description | White or cream-coloured granules or powder, odourless |
| 2. Identification (IR Spectrum) | Must conform with the Reference Standard Spectrum. |
| 3. pH (1% Solution) | Between 5.0 and 7.0 at 25 degrees C. |
| 4. Loss on Drying | NMT 10.0% at 102 degrees C. for 6 hours. |
| 5. Residue on Ignition | Between 15.0 and 19.0% |
| 6. Protein Content | NMT 0.10% |
| 7. Heavy Metals | NMT 20 ppm (as per USP 23 p. 1727). |
| 8. Arsenic | NMT 2 ppm (as per USP 23, p. 1724). |
| 9. Residual Solvents | a) Acetone: NMT 0.1%<br>b) Ethanol: NMT 2.0%<br>c) Formaldehyde: NMT 100 ppm |
| 10. Sodium Hyaluronate Assay | 97.0 to 102.0% (dried basis) |
| 11. Intrinsic Viscosity | Between 10.0 to 14.5 deciliters per gram. |
| 12. Molecular Weight<br>(calculated using the Laurent Formula) | Between 500,000 to 800,000 daltons<br>(based on intrincis viscosity). |
| 13. Total Aerobic Microbial Count | NMT 50 microorganism/gram (as per USP 23, p. 1684). |
| 14. Test for Escherichia coli | Escherichia coli is absent (as per USP 23, p. 1685). |
| 15. Yeasts & Molds | NMT 50 microorganisms/gram (as per USP 23, p. 1686). |
| 16. Endotoxins (LAL) | NMT 0.07 EU/mg (as per USP 23, p. 1696). |

A topical grade of hyaluronan may, in certain circumstances be suitable and may be made from the following granules/powder which have the following characteristics:

| Tests | Specifications |
|---|---|
| 1. Description | White or cream-coloured granules or powder, odourless |
| 2. Identification (IR Spectrum) | Must conform to the Reference Standard Spectrum. |
| 3. pH (1% Solution) | Between 6.0 and 8.0 at 25 degrees C. |
| 4. Loss on Drying | NMT 10.0% at 102 degrees C. for 6 hours. |
| 5. Residue on Ignition | Between 15.0 and 19.0% |
| 6. Protein Content | NMT 0.40% |
| 7. Heavy Metals | NMT 20 ppm (as per USP 23 p. 1727). |
| 8. Arsenic | NMT 2 ppm (as per USP 23, p. 1724). |
| 9. Residual Solvents | a) Acetone: NMT 0.1%<br>b) Ethanol: NMT 2.0%<br>c) Formaldehyde: NMT 100 ppm |
| 10. Sodium Hyaluronate Assay | 97.0 to 102.0% (dried basis) |
| 11. Intrinsic Viscosity | Between 11.5 to 14.5 deciliters per gram. |
| 12. Molecular Weight (calculated using the Laurent Formula) | Between 600,000 to 800,000 daltons (based on intrinsic viscosity). |
| 13. Total Aerobic Microbial Count | NMT 100 microorganism/gram (as per USP 23, p. 1684). |
| 14. Test for Staphylococcus aureus | Staphylococcus aureus is absent (as per USP 23, p. 1684). |
| 15. Test for Pseudomonas aeruginosa | Pseudomonas aeruginosa is absent (as per USP 23, p. 1684). |

| | |
|---|---|
| 16. Yeasts & Molds | NMT 200 CFU/gram (as per USP 23, p. 1686). |

This topical grade may then be sterilized.

Other forms of hyaluronic acid and/or its salts may be chosen from other suppliers, for example those described in prior art documents disclosing forms of hyaluronic acid having lower molecular weights between about 150,000 daltons and 750,000 daltons being prepared as for example, 1-2% solutions in sterile water for intravenous administration.

The following references teach hyaluronic acid, sources thereof and processes of the manufacture and recovery thereof.

Canadian Letters Patent 1,205,031 (which refers to United States Patent 4,141,973 as prior art) refers to hyaluronic acid fractions having average molecular weights of from 50,000 to 100,000; 250,000 to 350,000; and 500,000 to 730,000 and discusses processes of their manufacture

Where high molecular weight hyaluronic acid (or salts or other forms thereof) is used, it must, prior to use, be diluted to permit administration and ensure no intramuscular coagulation. Recently, it has been found that large molecular weight hyaluronic acid having a molecular weight exceeding about 1,000,000 daltons self-aggregates and thus, does not interact very well with HA receptors. Thus, the larger molecular weight hyaluronic acid should be avoided.

Briefly, the methods for linking or combining the hyaluronan targeting sequence onto other proteins using the hyaluronan binding motif (HBM), may be the methods outlined as follows or any other suitable methods as would be understood by persons skilled in the art:

A first method is recombinant technology which involves linking the HBM sequence to a DNA sequence encoding a therapeutic protein. The whole recombinant DNA is then translated by bacteria to make an artificial protein that is used for therapy. In this regard, see "Identification of Two Hyaluronan-binding Domains in the Hyaluronan Receptor", RHAMM, *The Journal of Biological Chemistry,* April 25, 1993, Vol. 268, No.12, pp. 8617 to 8623 previously discussed herein. In this report, the scientists (including me) have disclosed:

> "In the course of preparing RHAMM cDNAs that were defective in binding HA to be used for genetic studies, we have identified the carboxyl terminus as the HA binding region of RHAMM. We

sought to identify the precise motifs that contained hyaluronan binding activity within this region.

In this report we demonstrate that the RHAMM cDNA fusion protein retains its ability to bind to HA in two types of binding assays including a new transblot assay using bio-tinylated HA (20) and HA-Sepharose affinity chromatography. We have defined the HA-binding domain(s) on RHAMM as a 35-amino-acid region located near the carboxyl terminus of RHAMM. We show that two motifs within this region, containing 11 and 10 amino acids, respectively, represent the HA binding motifs of RHAMM. Neither of these motifs nor the entire 35-amino-acid region containing these motifs bears any amino acid sequence homology to other characterized hyaluronan-binding proteins."

In the Experimental Procedures, the scientists disclosed the following:

"Construction of Recombinant RHAMM-containing Oligonucleotides Encoding HA-Binding Peptides--PCR was used to incorporate the HA binding regions (peptide$^{aa401-411}$ and peptide$^{aa423-432}$, respectively)into a cDNA encoding the $NH_2$ terminus of RHAMM that was prepared as a 0.71-kb fragment (aa 1-238, see above and Fig. 2). The fusion protein product of this fragment did not have the ability to bind HA (Fig. 7). The procedure was carried out by making two PCR primers (5'TAG AAT GAA TTC TTT CAA TTT CAC AAC ATG TTT GAT TTT TTG TTT AAG ATC TTC TAT TTC *and* 5'TAG AAT GAA TTC TTT CCT TTT AAC AAG CTG AGA TCG CAG TTT AAG ATC TTC TAT TTC) which contained both a region mimicking the oligonucleotides encoding either peptide$^{aa401-411}$ or peptide$^{aa423-432}$ (creating an *Eco*RI site at the end of each primer) and a region mimicking 18 base pairs of the 3' end cDNA of the 0.71-kb insert. Recombinant cDNA was obtained with a PCR reaction by using either of these two primers together with a primer that mimicked the 5' end of the RHAMM cDNA (nucleotide 1-22) (creating a *Bam*HI site) with the same conditions described in the construction of RHAMM cDNA. Both PCR products were digested with *Eco*RI and *Bam*HI and purified in 1% agarose gel electrophoresis. Recombinant cDNAs were then inserted into pGEX-2T and

transformed into HB101 as above. The correct insertion of the recombinant cDNAs was confirmed by restriction endonuclease digestion of the selected clones and by sizing of the insert with agarose gel electrophoresis.

The major findings of this paper are that a critical interaction of hyaluronan with the RHAMM receptor can be localized to a region of 35 amino acids (aa 400-434) near the carboxyl terminus of this protein. This region contains sequences that exhibit clusters of basic amino acids. Peptides mimicking these sequences contain HA binding activity, and furthermore, these peptides confer HA binding activity to an $NH_2$-terminal fragment of RHAMM that does not bind to HA. Collectively, these results indicate that these sequences represent two critical HA binding motifs of RHAMM.

In the article *Identification of a common Hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein*, The EMBO Journal, Vol. 13, no. 2, PP. 286-296, 1994, the authors disclosed that:

> We have previously identified two hyaluronan (HA) binding domains in the HA receptor, RHAMM, that occur near the carboxyl-terminus of this protein. We show here that these two HA binding domains are the only HA binding regions in RHAMM, and that they contribute approximately equally to the HA binding ability of this receptor. Mutation of domain II using recombinant polypeptides of RHAMM demonstrates that K423 and R431, spaced seven amino acids apart, are critical for HA binding activity. Domain I contains two sets of two basic amino acids, each spaced seven residues apart, and mutation of these basic amino acids reduced their binding to HA-Sepharose. These results predict that two basic amino acids flanking a seven amino acid stretch [hereafter called B(X7)B] are minimally required for HA binding activity. To assess whether this motif predicts HA binding in the intact RHAMM protein, we mutated all basic amino acids in domains I and II that form part of these motifs using site-directed mutagenesis and prepared fusion protein from the mutated cDNA. The altered RHAMM protein did not bind HA, confirming that the basic amino acids and their spacing are critical for binding. A specific requirement for arginine or lysine residues was identified since

mutation of K430, R431 and K432 to histidine residues abolished binding. Clustering of basic amino acids either within or at either end of the motif enhanced HA binding activity while the occurrence of acidic residues between the basic amino acids reduced binding. The B(X7)B motif, in which B is either R or K and X7 contains no acidic residues and at least one basic amino acid, was found in all HA binding proteins molecularly characterized to date. Recombinant techniques were used to generate chimeric proteins containing either the B(X7)B motifs present in CD44 or link protein, with the amino-terminus of RHAMM (Amino acids 1-238) that does not bind HA. All chimeric proteins containing the motif bound HA in transblot analyses. Site-directed mutations of these motifs in CD44 sequences abolished HA binding. Collectively, these results predict that the motif of B(X7)B as a minimal binding requirement for HA in RHAMM, CD44 and link protein, and occurs in all HA binding proteins described to date.

The protein -HBM may then be combined with hyaluronan and will become bound thereto.

A second method is to prepare the HA targeting sequence by synthesis (a standard procedure as would be understood by persons skilled in the art) and then link to a protein via carbodiimide linkage. This is a chemical method for linking carboxyl and amino groups together. Such a procedure is described in Spontaneous Glycosylation of Glycosaminoglycan Substrates of Adherent Fibroblasts, *Cell*, May 1979, Vol. 17, 109-115 by E.A. Turley and S. Roth. At page 114, the following experimental procedure is found:

> **"Derivatization and Characterization of Glycosaminoglycan Dishes"**
>
> The glycosaminoglycans, chondroitin-6-sulfate (Type C, Sigma; molecular weight 50,000), hyaluronic acid (bovine vitreous humor, Sigma; molecular weight 1,000,000) and polygalacturonic acid (Sigma; molecular weight 20,000-400,000), were covalently linked to 35 x 10mm polystyrene tissue culture dishes (Falcon Plastics) by a modification of the procedure of Kenner, McDermott and Sheppard (1971). Each dish was treated for 1 hr with 1 ml of concentrated sulfuric acid at 37°C, washed extensively with water and then treated with 1 ml of aqueous ammonium hydroxide (30%, v/v) at room temperature for 24 hr. The resulting polysulfonamide dishes were incubated with 1 ml of an aqueous solution of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (25 mg/ml) and either chondroitin

sulfate (CS dishes), hyaluronic acid (HA dishes) or polygalacturonic acid (PGA dishes) all at 5 mg/ml for 48 hr in a humidified atmosphere at 37°C. Dishes were then boiled in 8 M urea and 10% sodium dodecylsulfate (SDS-urea) for 20 min to remove noncovalently bound material. Dishes were pulverized, and hydrolysis of bound sugars was achieved with 1.5 ml of 90% formic acid (v/v) at 105°C for 6-12 hr. Hydrolysates were lyophilized and the residue was dissolved in 500 µl water. This solution was assayed for uronic acid (Dische, 1947) in the case of HA and PGA dishes, or counted in a Searle Mark III liquid scintillation counter in the case of CS dishes derivatized with $^{3}$H-chondroitin sulfate (3nmole per plate; spec. act. 1.5 x $10^{8}$ cpm/mg). Aliquots of all samples were analyzed by high voltage electrophoresis (50 V/cm for 45 min) on Whatman 3MM paper impregnated with 1% borate (w/v). For standards, D-U-$^{14}$C-glucuronic acid (8 nmole, spec. act. 76mCi/nmole; Amerisham-Searle), hyaluronic acid, chondroitin sulfate and polygalacturonic acid were all hydrolyzed under identical conditions. The unlabeled hexosamine standards were visualized on paper using periodate-benzidine (Smith, 1969)."

Once again the protein-HBM is then combined with hyaluronan.

A third method is simply to synthesize the therapeutic peptide and HA targeting sequence (HBM) together by standard peptide synthesis known to persons skilled in the art. This could be done for example, if the disease modifier (therapeutic agent) were a peptide of for example, 10-20 amino acids. This procedure would make economic sense rather than using bacteria to make it as a small protein. The product is then combined with hyaluronan. The new compounds formed by interposing the hyaluronan binding motif (HBM) between the disease modifier (e.g. drug) and hyaluronan, may be administered in the usual manner as one administers the hyaluronan or disease modifier either together or individually.

Because toxicity may be associated with the disease modifier, toxicity will have to be considered in the amounts bound through the HBM to the hyaluronan. However because of the improvement in delivery by my invention, less of the disease modifier may be required (than would be used normally to treat the disease or condition) and therefore toxicity concerns are less. Further where dosages of the hyaluronan exceed 200 mg per person (for example a 70 kg person), side effects attributed to the drug modifier may be reduced such as gastrointestinal distress, neurological abnormalities, depression, etc.

The invention may thus be used to bind hyaluronan through HBM to a protein or peptide. For example the protein tissue inhibitors of metalloproteinases (TIMPS) which break down collagen can be made recombinantly. HA binding motif (HBM) may be added to TIMPS in one of the many known manners and the product can be combined with hyaluronan to form the dosage amount to be administered to a patient. The amounts of TIMPS and HA are chosen in amounts suitable for use to treat a patient in need of treatment. When administered to the patients (for example by injection), the HA bound TIMPS goes to the site of injury (the pathological tissue site which expresses a surplus of HA receptors) for treatment of the injury. The invention can be used for administration of any protein disease modifier.

Thus the invention can be used to target disease modifiers which are proteins such as recombinant proteins or peptides such as TIMPS, enzymes, collagenese, cytokines, growth facts, therapeutic proteins (such as antibiotics which may be proteins).

Figures 1, 2, 3 and 4 are provided illustrating methods of binding proteins to hyaluronan binding motifs.

Figure 1 relates to the competition and direct binding assays of synthetic peptides corresponding to positive charge clusters in RHAMM protein. Panel A, transblotted RHAMM fusion protein was stained with a 1:3,000 dilution of biotin-labeled HA that had been preincubated for 1 h with 3 mg/ml bovine serum albumin (BSA) (lane 1), 3 mg/ml peptide$^{aa401-411}$ (lane 2), or 3 mg/ml peptide$^{aa423-432}$ (lane 3). Both peptides significantly reduced the binding of HA to RHAMM fusion protein. Panel B, HA-Sepharose affinity gel was prepared according to the manufacturer's instruction/ RHAMM peptides (peptide$^{aa401-411}$; peptide$^{aa423-432}$; randomized peptide$^{aa401-411}$ (LKQKKVKKHIV); randomized peptide$^{aa423-432}$ (QSKRLKKRVL); peptide$^{aa125-145}$ and peptide$^{aa269-288}$, 20) were applied to HA-Sepharose. Unbound peptides were removed by washing the gel with PBS containing 0.15 M NaCl. The amounts of peptides applied and the unbound peptide removed from the gel were determined by measuring their OD value. The results indicated that peptide$^{aa401-411}$ and peptide$^{aa423-432}$ bound in highest amounts to HA-Sepharose gel.

Figure 2 relates to construction of recombinant RHAMM containing HA binding domains. Panel A, cDNA encoding peptide$^{aa401-}$

411 (*) and peptide$^{aa423-432}$ (•) were, respectively, aligned by PCR to a cDNA encoding RHAMM $NH_2$-terminal polypeptide$^{aa1-238}$ that did not have the ability to bind HA. This was carried out as described under "Experimental Procedures." Both PCR products were digested with *Eco*RI and *Bam*HI and purified with agarose gel electrophoresis. The cDNAs were inserted into pGEX-2T opened with *Bam*HI and *Eco*RI, which were cloning sites that were followed by stop codons, and transformed into HB101. The correct inserts were confirmed by restriction endonuclease digestion of the selected clones and were expressed as glutathione S-transferase-RHAMM fusion proteins. Panel B, bacterial cell lysates containing the glutathione S-transferase-RHAMM fusion proteins were fractionated on SDS-PAGE, transblotted onto nitrocellulose membrances, and visualized with either polyclonal antibody to peptide$^{aa125-145}$ (lanes 1-3) or biotin-labeled HA (lanes 4-6). The glutathione S-transferase fusion non-recombinant polypeptide$^{aa1-238}$ was used as a control (lanes 1 and 4). The linkage of either peptide$^{aa401-411}$ (lanes 2 and 5) or peptide$^{aa423-432}$ (lanes 3 and 6) to the $NH_2$-terminal RHAMM polypeptide$^{aa1-238}$ created HA-binding domains (lanes 5 and 6) although their antibody binding properties remained the same (lanes 2 and 3).

Figure 3 relates to the deletion and mutation of HA binding domains in RHAMM. (A) The HA binding domain II (aa423-432) was completely deleted and the HA binding domain I (aa 401-411) was partially deleted. The remaining domain (aa 401-411) was altered by mutating K405 and K409 to E. (B) The PCR product (Figure 2A, lane 2) was ligated into the plasmid-containing fragment (5.3 kb in lane 3) and transformed into *E.coli* HB101. The clone containing the correct insert (lane 4) was used to prepare RHAMM fusion protein. (C) Cell lysates containing the complete fusion proteins (lanes 2 and 5), deleted fusion protein (lanes 1 and 4) and HB101 lysate (lanes 3 and 6) were prepared by sonication, then separated by SDS-PAGE and immunoblotted. RHAMM protein was visualized using anti-RHAMM antibody (lanes 1-3) or biotin-labelled HA (lanes 4-6). The results show that after deletion of domains I and II, RHAMM lost its ability to bind to biotin-labelled HA (lane 4). The bacterial lysate contains an HA binding protein of <26 kDa that is not related to RHAMM (lanes 4-6).

Figure 4 relates to the strategy for defining the critical basic amino acids that determine the HA binding properties of domain II. To

investigate the basic amino acids required in domain II for HA binding, six independent mutations were carried out and a RHAMM fusion peptide was genesuted recombining domain II with the amino-terminus (aa 1-238) of RHAMM using a recombinant technique. (A) The primers used to generate the altered cDNAs. (B) The resultant amino acid sequences. Highlighted amino acids indicate mutations. Six cDNAs, each containing site-directed mutation(s) were generated in the PCRs diagrammed in panels A and B, using RHAMM cDNA$^{1-720}$ as the template DNA and containing the oligonucleotides encoding aa 423-432 of RHAMM with different mutations. PCR products from the six primers containing the mutated nucleotides were doubly digested with *Bam*HI+*Eco*RI, ligated into pGEX-2T and transformed into HB101. Selected clones were confirmed to contain correct inserts by double digestion with *Bam*HI+*Eco*RI and electrophoresis on agarose gels. Fusion proteins were prepared from clones and analyzed in Western blots with either anti-RHAMM antibody to visualize RHAMM protein (C) or biotin-labelled HA (D) to assay HA binding activity. The results show that the HA binding ability of mutations I-VI (panel D) was reduced to 0, 67, 38, 21, 2 and 40%, of the control (lane 1), respectively. Lane 1, control; lane 2, mutation I; lane 3, mutation II; lane 4, mutation III; lane 5, mutation IV; lane 6, mutation V; lane 7, mutation VI.

The amounts of disease modifiers and form of hyaluronan may be those previously used even band together through HBM. Because of the beneficial effects of the form of hyaluronan taking the disease modifier to the pathological tissue (having excess HA receptors) in need of treatment, less of the disease modifier than would normally be expected to be used may be useful to treat and resolve the condition/disease affecting the pathological tissue. The amounts of the forms of hyaluronan may be those amounts specified in WO91/04058 -- at least about 10mg of the form of hyaluronan in each dosage amount to in excess of 1000-1500mg of the form of hyaluronan in each dosage amount administered to a patient.

As many changes could be made to the examples without departing from the scope of the invention, it is intended that all material herein be interpreted as illustrative of the invention and not in a limiting sense.

**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS BEING CLAIMED ARE AS FOLLOWS:**

1. A pharmaceutical composition component comprising hyaluronan binding motif (HBM) interposed between a form of hyaluronan having a molecular weight (protein standard) less than 750,000 daltons and a disease modifier which comprises a peptide or protein.

2. The pharmaceutical composition component of claim 1 wherein flanking basic amino acids are added to HBM to enhance the affinity of the hyaluronan for the HBM.

3. The pharmaceutical composition component of claim 1 wherein several internal basic amino acids are added at position 4,5 to HBM to enhance the affinity of the hyaluronan for the HBM.

4. The pharmaceutical composition component of claim 1 wherein intervening amino acids are hydrophilic and not acidic.

5. The pharmaceutical composition component of claim 3 wherein intervening amino acids are hydrophilic and not acidic.

6. A pharmaceutical composition comprising an effective amount of the pharmaceutical composition component of claim 1, 2, 3, 4 or 5 together with suitable pharmaceutical excipients.

7. The pharmaceutical composition of claim 6 wherein the amount of hyaluronan exceeds 10mg.

8. The pharmaceutical composition of claim 7 wherein the amount of hyaluronan is less than 3000mg.

9. A method of treating a patient comprising administering to a patient at least or pharmaceutical composition component as claimed in claim 1, 2, 3, 4 or 5 for such period of time as required.

10. A method of treating a patient comprising administering to a patient at least a pharmaceutical composition as claimed in claim 6, 7 or 8 for such period of time as required.

11. The pharmaceutical composition component of claim 1, 2, 3, 4 or 5 wherein the disease modifier is selected from a cytokine, a peptide mimicking a cytokine, a protein mimicking a cytokine.

12. The pharmaceutical composition of claim 6, 7 or 8 wherein the disease modifier is selected from a cytokine, a peptide mimicking a cytokine, a protein mimicking a cytokine.

13. The method of claim 9 or 10 wherein the disease modifier is selected from a cytokine, a peptide mimicking a cytokine, a protein mimicking a cytokine.

14. A method of protecting a disease modifier which comprises a protein or peptide when administered to a patient from attack from proteases comprising administering to the patient an effective amount of the disease modifier which is bound through HBM to a form of hyaluronan according to claim 1, 2, 3, 4 or 5 which is in an effective amount to protect the disease modifier from attack from proteases.

15. A method of protecting a disease modifier which comprises a protein or peptide when administered to a patient from the patient's immune system recognition and attack comprising administering to the patient an effective amount of the disease modifier which is bound through HBM to a form of hyaluronan according to claim 1, 2, 3, 4 or 5 which is an effective amount to protect the disease modifier from immune system recognition and attack.

16. The pharmaceutical composition component of any previous pharmaceutical composition component claim wherein the form of hyaluronan is sodium hyaluronate.

17. The pharmaceutical composition of any previous pharmaceutical composition claim wherein the form of hyaluronan is sodium hyaluronate.

18. The method of any previous method claim wherein the form of hyaluronan is sodium hyaluronate.

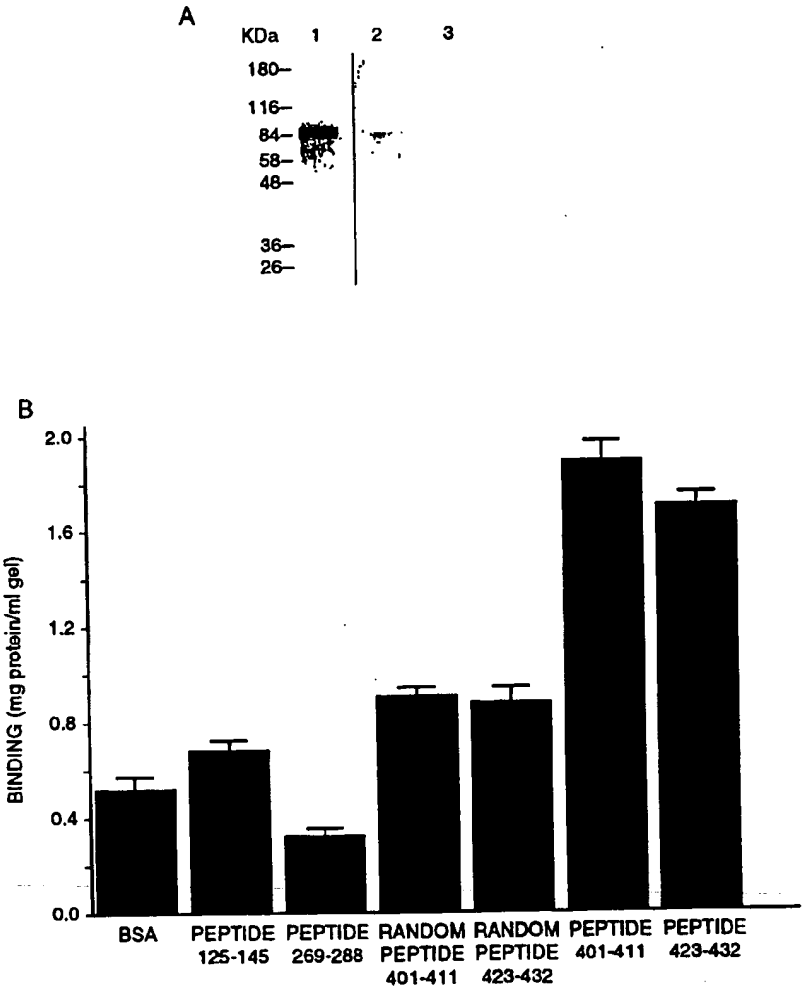

A
KDa
1
2
3
180–
116–
84–
58–
48–
36–
26–
B
BINDING (mg protein/ml gel)
2.0
1.6
1.2
0.8
0.4
0.0
BSA
PEPTIDE 125-145
PEPTIDE 269-288
RANDOM PEPTIDE 401-411
RANDOM PEPTIDE 423-432
PEPTIDE 401-411
PEPTIDE 423-432

**Figure 1**

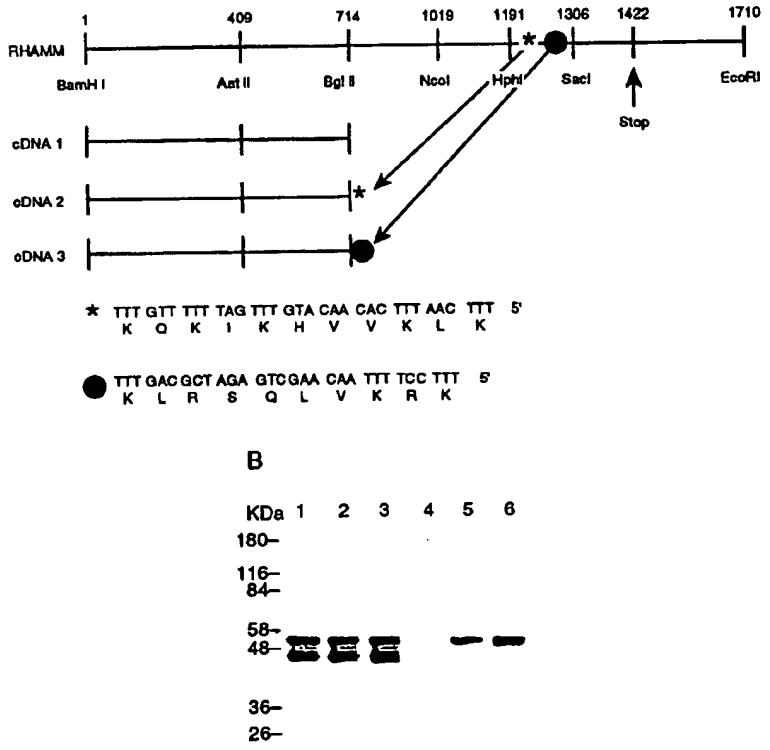

1
409
714
1019
1191
1306
1422
1710
RHAMM
BamH I
Ast II
Bgl II
NcoI
HphI
SacI
Stop
EcoRI
cDNA 1
cDNA 2
cDNA 3
* TTT GTT TTT TAG TTT GTA CAA CAC TTT AAC TTT 5'
K Q K I K H V V K L K
TTT GAC GCT AGA GTC GAA CAA TTT TCC TTT 5'
K L R S Q L V K R K
B
KDa 1 2 3 4 5 6
180–
116–
84–
58–
48–
36–
26–

**Figure 2**

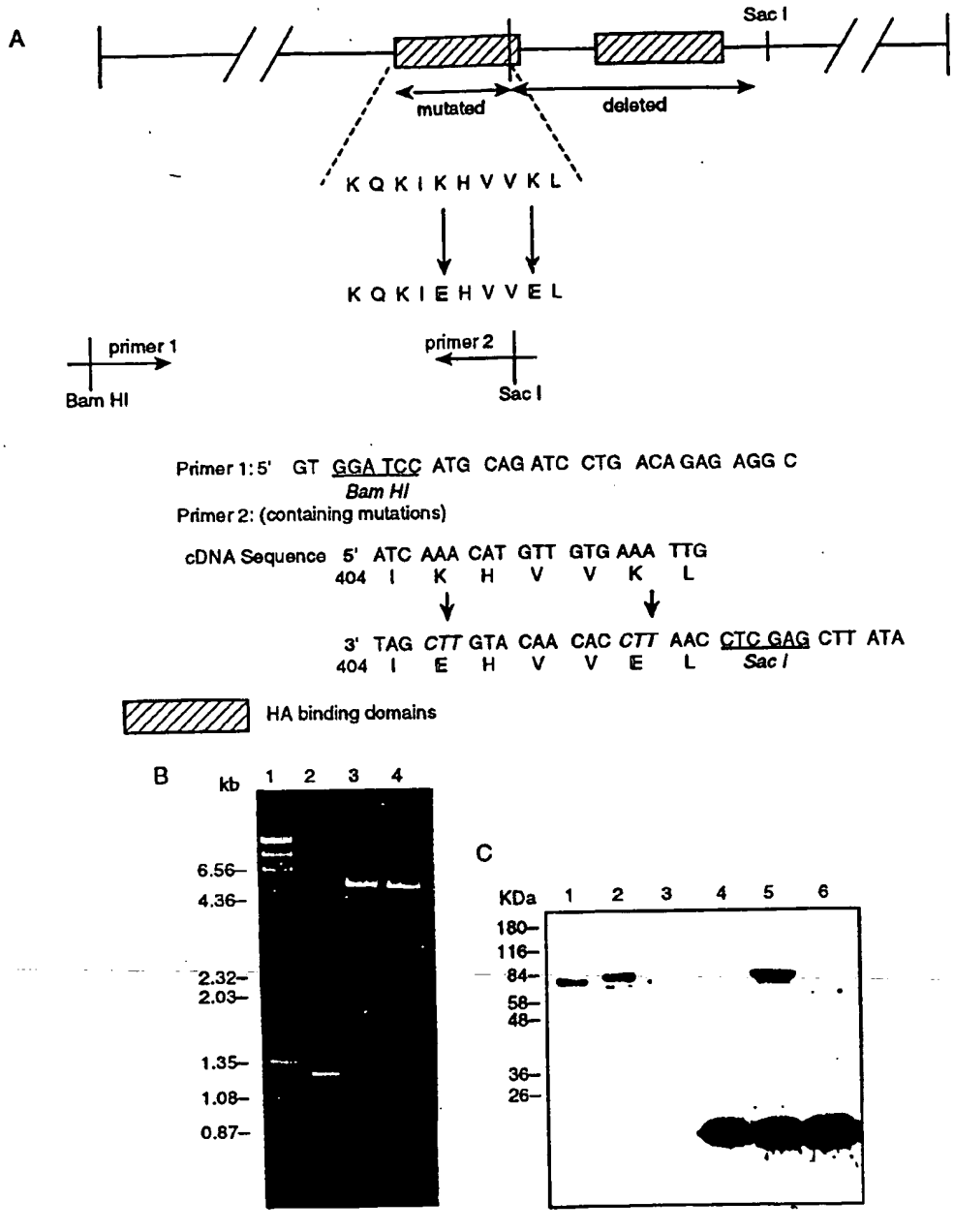
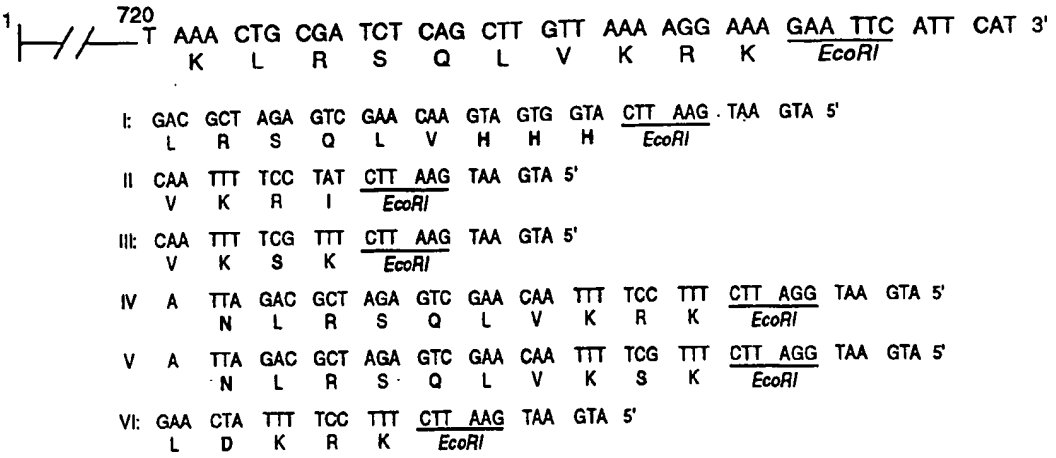

A
Sac I
mutated
deleted
K Q K I K H V V K L
K Q K I E H V V E L
primer 1
Bam HI
primer 2
Sac I
Primer 1: 5' GT GGA TCC ATG CAG ATC CTG ACA GAG AGG C
Bam HI
Primer 2: (containing mutations)
cDNA Sequence 5' ATC AAA CAT GTT GTG AAA TTG
404 I K H V V K L
3' TAG CTT GTA CAA CAC CTT AAC CTC GAG CTT ATA
404 I E H V V E L Sac I
HA binding domains
B
kb 1 2 3 4
6.56
4.36
2.32
2.03
1.35
1.08
0.87
C
KDa 1 2 3 4 5 6
180
116
84
58
48
36
26

Figure 3

A Site-directed mutations of peptide 423-432:




B Resultant amino acid sequences:

| | |
|---|---|
| Peptide 423-432: | K L R S Q L V K R K |
| Mutation I: | K L R S Q L V H H H |
| Mutation II: | K L R S Q L V K R I |
| Mutation III: | K L R S Q L V K S K |
| Mutation IV: | N L R S Q L V K R K |
| Mutation V: | N L R S Q L V K S K |
| Mutation VI: | K L R S Q L D K R K |

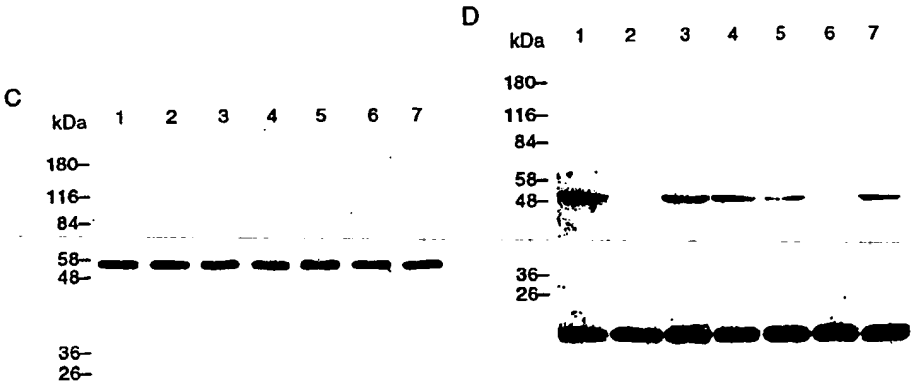



Figure 4